Increased Risk of Hepatocellular Carcinoma in Male Hepatitis B Surface Antigen Carriers With Chronic Hepatitis Who Have Detectable Urinary Aflatoxin Metabolite M1

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We followed 145 men with chronic hepatitis B virus (HBV) hepatitis for 10 years to determine whether exposure to aflatoxin, or concomitant exposure to hepatitis C virus (HCV), or family history of hepatocellular carcinoma (HCC) increased the risk of developing HCC. We collected 8 monthly urine samples before beginning follow-up and pooled them to detect aflatoxin metabolite M1 (AFM1). AFM1 was detected in 78 (54%) of the subjects. The risk of HCC was increased 3.3-fold (with a 95% confidence interval of 1.2-8.7) in those with detectable AFM1 (above 3.6 ng/L). This relative risk was adjusted for age and for HCV status. The attributable risk from exposure to detectable AFM1 was 0.553 (0.087, 0.94). The relative risk of fatal cirrhosis for those with elevated AFM1 was 2.8 (0.6, 14.3), and the odds of having a persistently elevated alanine transaminase (ALT) were 2.5-fold greater in those with detectable AFM1 ($P = .007$). Concomitant infection with HCV increased the risk of HCC 5.8-fold (2.0-17), adjusted for age and AFM1 status. A family history of HCC increased the risk of HCC 5.6-fold, adjusted for age and AFM1. Four men with detectable AFM1 and HCC all had missense mutation in codon 249 of the p53 gene in cancer tissues. This study shows that exposure to AFM1 can account for a substantial part of the risk of HCC in men with chronic HBV hepatitis and adds importantly to the evidence that HCV and family history of HCC increase the risk of HCC in men with chronic HBV hepatitis. (HEPATOLOGY 1999;30:379-383.)

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; AFM1, metabolite M1 of aflatoxin; HCV hepatitis C virus; ALT, alanine transaminase; HPLC, high-pressure liquid chromatography.

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Hepatocellular carcinoma (HCC) is the second most common cause of death from cancer in China, where the mortality rate was 18 per 100,000 person-years in 1990 through 1992.[1] Infection with hepatitis B virus (HBV) and exposure to aflatoxin are both important risk factors for HCC. Ross et al.[2] studied 18,244 men aged 45 to 64 who lived in Shanghai between 1986 and 1989. In a sample of 140 controls who were age-matched to HCC cases, it was found that 15 (11%) were hepatitis B surface antigen (HBsAg)-positive, and 53 (38%) had detectable urinary aflatoxin metabolites or DNA-adducts. A later analysis of 50 HCC cases and 267 age-matched controls from this study[3] showed that compared with men without HBsAg or urinary aflatoxin biomarkers, relative risks were 7.3 with 95% a confidence interval (2.2, 24) for those only with HBsAg, 3.4 (1.1, 10) for those only with aflatoxin biomarkers, and 59 (17, 212) for those with both. Based on such data, Ross et al.[2] and Qian et al.[3] suggested that reduction of exposure to aflatoxin might prevent a considerable fraction of the HCC in this population.

The previous cohort represented the general male population of Shanghai. We chose instead to follow prospectively a representative group of 145 carriers of HBsAg with a history of chronic hepatitis. These men were being cared for at the Medical Oncology Department of the Qidong Liver Cancer Institute/Hospital, Qidong, Jiangsu Province, China. HCC rates are very high in Qidong. The purpose of the study was to determine whether exposure to aflatoxin increased the risk of HCC or of fatal cirrhosis over a 10-year period in patients with HBV hepatitis. A positive finding would suggest that measures to reduce exposure to aflatoxin might also be beneficial to men with chronic HBV hepatitis and could be evaluated in treatment protocols. Because we collected monthly urine samples for 8 months before beginning follow-up, we were able to pool the samples to obtain estimates of long-term average urinary aflatoxin M1 (AFM1) concentrations, and the assay could detect concentrations of AFM1 as low as 3.6 ng/L. These data also give information on the added risks of HCC in men with chronic HBV hepatitis from exposure to hepatitis C virus (HCV) and from a family history of HCC.

PATIENTS AND METHODS

Population-based sampling was used to obtain a representative study cohort. In an earlier study of the prevalence of HBV infection sponsored by the Ministry of Public Health, China, from 1981 to 1982, 2 townships, formerly called communes, were selected at random from among 45 townships in Qidong. These 2 townships

contained approximately 50,000 inhabitants, of whom approximately 12,500 were men aged 25 to 60. These men were principally farmers of Han ancestry. All the men consented to screening for HBV in the earlier study. About 16% of these men had detectable HBsAg, and about 10% of those with detectable HBsAg had chronic hepatitis defined by persistent HBsAg antigenemia, elevations in serum alanine transaminase (ALT) on 2 occasions at least 6 months apart, and evidence of hepatomegaly or other hepatic abnormalities. Men seen at the Medical Oncology Department of the Qidong Liver Cancer Institute/Hospital who satisfied these criteria for chronic HBV hepatitis were offered a chance to register for the present study. Of the first 150 men to register, 145 were enrolled because they satisfied the criteria above and were deemed healthy enough for long-term follow-up. We estimate that these 145 men constitute about 72% of the roughly 12,500 × 16% × 10% = 200 men with chronic hepatitis in these 2 townships. Because the townships were selected at random, the screening participation rates were virtually 100%, the only persons eligible for treatment at the Medical Oncology Department of the Qidong Liver Cancer Institute/Hospital are from Qidong, and the patients were recruited on a first-come-first-enrolled basis, the study population is quite representative.

The men in the study were recruited from July 31, 1987, to July 31, 1998, and 8 100-mL urine samples were obtained at monthly intervals and stored at −25°C. Baseline data were obtained on family history, alcohol consumption, cigarette consumption, and HCV status. Study participants were examined and blood was drawn once or twice a year at the Liver Cancer Institute/Hospital during the prospective follow-up from August 1, 1988, to July 31, 1998. Serum alanine transaminase (ALT) was measured with a Beckman Biochemical Analyzer, and levels were defined as "persistently elevated" if the level exceeded 40 international units/mL on at least 2 occasions in separate years. The diagnosis of HCC was made on the basis of clinical criteria alone (progressive hepatomegaly, rising pattern of serum α-fetoprotein, ultrasonograms showing space-occupying lesions) in 12 cases, and confirmed by pathological diagnosis in 10 other cases. Severe fatal cirrhosis was diagnosed in 10 cases on the basis of clinical history and death-certificate data. To measure baseline AFM1, we pooled the urine samples and filtered and immuno-concentrated about 350 mL of pooled urine.[4,5] The concentrate was eluted and analyzed by high-pressure liquid chromatography (HPLC) using both a Gilson HM/HPLC (365 nm) ultraviolet detector and a Specto-Glo fluorescence detector. Standard AFM1 (Sigma) was used to demonstrate a detectability limit of 0.5 ng, which corresponds to a detectabilty threshold of AFM1 in urine of 3.6 ng/L based on a recovery fraction of 40%. In particular, our estimates of AFM1 excretion were obtained by dividing the amount of AFM1 detected per liter of urine by 0.4, and we defined the AFM1 level as "detectable" if it exceeded 3.6 ng/L. AFM1 analysis by HPLC and serum HBsAg and anti-HCV enzyme-linked immunosorbent immunoassays with kits from Abbott were performed in the Cancer Institute/Hospital of the Chinese Academy of Medical Sciences in Beijing.

Four HCC surgical samples in paraffin blocks were available for DNA extraction. The DNA fragments spanning the seventh exon of the p53 gene were amplified by polymerase chain reaction using primer sets that were previously reported.[6] The mutational status of the 249 codon of p53 was identified by restriction fragment pattern following *Hae*III enzyme digestion.[7]

Event rates (Table 1) were calculated as the number of events divided by the person-years exposure. Follow-up began on August 1, 1988, and ended at the earliest of the times of the event, death, or July 31, 1998. To compute significance probabilities for trend tests in Table 1, we assumed that event counts had a Poisson distribution, so that, conditional on the total count, the event counts were multinomial with cell probabilities determined by person-years exposure. Assigning scores 0, 1, or 2 for 3 levels or 0 and 1 for 2 levels of a covariate, we computed the exact conditional null distribution of T = the sum of (score times the number of events), where the sum is over levels of the covariate. The *P* value was calculated as the null probability of a value of T as great or greater than the observed value or less than the null mean minus the observed value. The proportional hazard model of Cox[8] was used for multivariate adjustment of relative risks. The time scale was usually taken to be time since August 31, 1988, but in special analyses, we used age as the time scale and adapted the analysis to accommodate left truncation.[9] In some analyses, age was grouped by tertiles into age ranges 0-34, 35-42, and ≥43 years. Assuming a known proportion exposed, we calculated the variance of the attributable risk by a Taylor series expansion in the hazard rates of HCC for those with and without detectable AFM1. A logit transformation was used to construct a confidence interval for the attributable risk.

TABLE 1. Rates Per 100 Person-Years of HCC and Fatal Cirrhosis

	HCC			Fatal Cirrhosis		
Factor (sample size)	No. of Events	Rate	Trend Test	No. of Events	Rate	Trend Test
Age (yr)						
0-34 (51)	6	1.26		1	0.21	
35-42 (45)	7	1.80	$P = .31$	4	1.02	$P = .40$
≥43 (49)	9	2.22		3	0.72	
AFM1 (ng/L)						
0-2.5 (67)	5	0.80		2	0.32	
2.6-30.9 (40)	10	3.17	$P = .073$	3	0.93	$P = .29$
≥31 (38)	7	2.14		3	0.91	
AFM1 (ng/L)						
0-3.6 (67)	5	0.80		2	0.32	
≥3.7 (78)	17	2.64	$P = .017$	6	0.92	$P = .29$
HCV						
Negative (135)	17	1.41		7	0.58	
Positive (10)	5	8.13	$P = .0035$	1	1.44	$P = .36$
Family history of HCC						
Negative (122)	13	1.18		7	0.63	
Positive (23)	9	5.26	$P = .0014$	1	0.55	$P = 1.00$
Smoker						
No (58)	11	2.27		3	0.61	
Yes (77)	11	1.59	$P = .52$	4	0.57	$P = 1.00$
Alcohol consumption						
<1,000 mL/mo (83)	16	2.30		5	0.70	
≥1,000 mL/mo (52)	6	1.25	$P = .28$	2	0.42	$P = .71$

RESULTS

No patients were lost to follow-up. The mean age of the population was 39.2 years as of August 1, 1998, with a standard deviation of 10.6 years. The mean age of the 78 AFM1-positive (>3.6 ng/L) subjects was 40.2 years (SD: 11.8), and was not significantly different from the mean age of the 67 AFM1-negative subjects 38.1 (SD: 9.1) based on a 2-tailed *t* test ($P = .23$). AFM1 was elevated above 3.6 ng/L in 78 (54%) of the subjects. There were 10 (6.9%) HCV-positive men, and 23 (16%) had a family history of HCC. Of these men, 21 (14%) had a history of HCC in first-degree relatives, and 2 had a history of HCC in 2 uncles. AFM1 levels are not strongly correlated with age (Fig. 1), and, indeed, the highest levels of AFM1 seem to occur in men in the age range of 30 to 45. The median level of AFM1 was 9.6 ng/L, and the highest level was 243 ng/L. Men who subsequently developed HCC (indicated by solid or open diamonds) tended to have increased AFM1 levels. Elevations of AFM1 were found in 6 of 10 men with HCV (solid circles or diamonds) compared with 72 of 135 (53%) of men without HCV. AFM1 was elevated in 4 of 6 men with HCV who developed HCC.

Rates of development of HCC per 100 person-years of follow-up suggest a trend with increasing age, but, perhaps

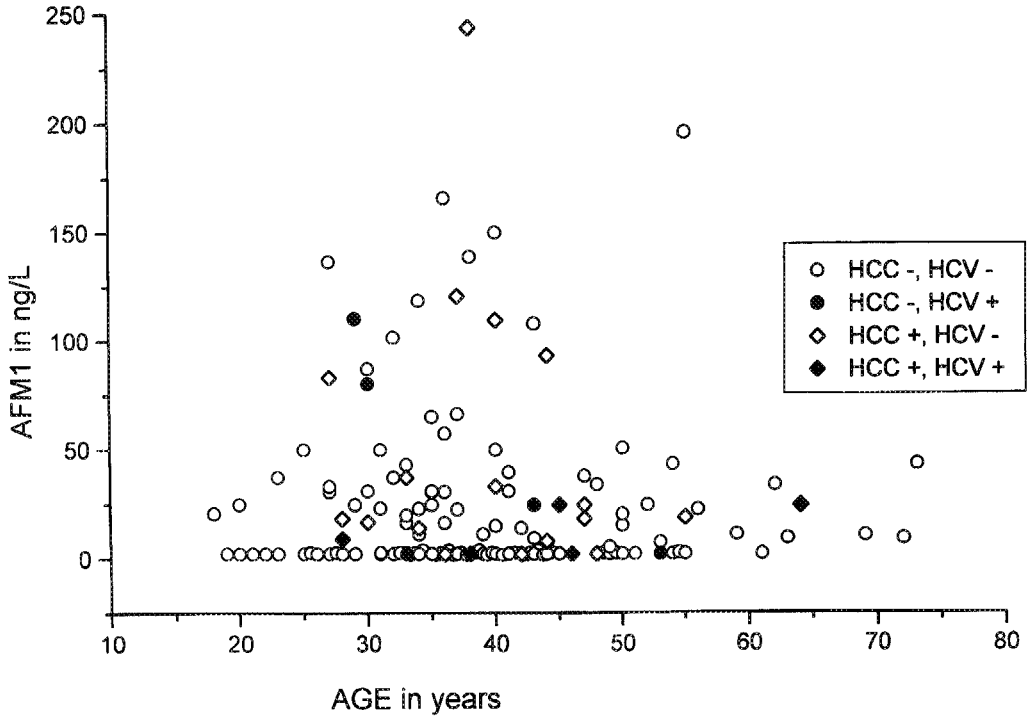

FIG. 1. Plot of urinary AFM1 concentration versus age, with separate symbols to denote the presence or absence of hepatocellular carcinoma and of antibody to HCV.

because there were only 22 HCC events, the trend was not statistically significant (Table 1). Increased levels of AFM1 are associated with increased HCC risk, and if the AFM1-negative group is compared with the AFM1-positive (>3.6 ng/L) group, the relative risk is 2.64/0.80 = 3.30 and is significantly greater than unity (P = .017). The fraction of HCC incidence accounted for by detectable AFM1 in this population, namely the attributable risk, is calculated from data in Table 1 as $1 - 0.80/[(74/145) \times 2.64 + (71/145) \times 0.80] = 0.553$, with a 95% confidence interval (0.087, 0.94). Thus, these data, though subject to large random uncertainty, suggest that about half the HCC incidence might be prevented by reducing exposure to aflatoxin to undetectable levels (urinary AFM1 below 3.7 ng/L).

Four available surgical samples of HCC in paraffin blocks, all from the AFM1-detectable group, consistently exhibited a missense mutation at the third base of codon 249 in exon 7 of the p53 gene. No other specimens were available for study, because most pathological diagnoses of HCC were based on postmortem needle biopsies at local township clinics that did not retain the specimens.

Those infected with HCV had 8.13/1.41 = 5.77 times the risk of HCC of those not infected with HCV, and this relative risk differs from unity (P = .0035). Men with a positive family history of HCC (21 men with affected first-degree relatives and 2 with 2 affected uncles) had a 5.26/1.18 = 4.46 times higher risk of HCC than men with no family history of HCC.

There were no statistically significant associations between HCC incidence and the consumption of more than 1,000 mL/mo of alcohol or with cigarette smoking in this population.

Similar trends with AFM1 were seen for rates of fatal cirrhosis (Table 1), but there were only 8 cases of fatal cirrhosis; thus, the numbers are too small to yield good power to obtain statistically significant results. There were no statistically significant associations between the incidence of fatal cirrhosis and consumption of more than 1,000 mL/mo of alcohol, cigarette smoking, or a family history of HCC. We used the Cox proportional hazards model[8] with stratification on age (0-34, 35-42, 43) and adjustment for HCV status to determine whether these factors accounted for the crude relative risk of HCC associated with elevated AFM1 (Table 2). Adjustments for age at entry into the cohort on August 1, 1988, or HCV status have little effect on the relative risk of HCC associated with elevated AFM1. The elevated risk for AFM1 also remains after adjustment for age, HCV status, and family history of HCC (Table 2). Similar results were obtained using age as the time scale in the Cox model (data not shown). AFM1 also appears to be associated with about a

TABLE 2. Multivariate Relative Risk Models (With 95% Confidence Intervals) for HCC and Fatal Cirrhosis

Outcome	AFM1 >3.6 ng/L	HCV-Positive	Family History of HCC
HCC	3.33 (1.23, 9.02)*		
		5.87 (2.15, 16.0)*	
			4.52 (1.93, 10.6)*
	3.29 (1.21, 8.94)		
		6.04 (2.10, 17.3)	
			4.70 (1.99, 11.1)
	3.20 (1.17, 8.72)	5.82 (1.99, 17.0)	
	3.96 (1.43, 10.9)		5.61 (2.36, 13.4)
		6.88 (2.36, 20.0)	5.24 (2.16, 12.7)
	4.52 (1.57, 13.0)	8.05 (2.68, 24.1)	7.13 (2.85, 17.8)
Fatal cirrhosis			
	2.84 (0.57, 14.1)*		
	2.94 (0.59, 14.7)		

NOTE. Each row represents a different statistical model with regressor variables that correspond to column entries.

*No stratification on age; in all other cases, we stratify on age in tertiles (18-34, 35-42, 43-73).

2.8-fold increased risk for fatal cirrhosis, but this association is not statistically significant, perhaps because there were only 8 cases of fatal cirrhosis.

Elevated AFM1 is associated with persistent elevations in ALT, both for HCV-negative and HCV-positive patients (Table 3). The Mantel-Haenszel estimate of the summary odds ratio is 2.53, and the corresponding significance test for no association yields $P = .007$. This association between baseline AFM1 and persistent elevations in ALT lends support to the hypothesis that elevated AFM1 can prolong or intensify chronic active hepatitis and accelerate progression to fatal cirrhosis.

The data in Table 2 also indicate that HCV and family history are associated with increased HCC risk, even after adjusting for age and AFM1 exposure.

DISCUSSION

This prospective cohort study demonstrated that detectable urinary AFM1 levels above 3.6 ng/L were associated with increased risk of HCC in male HBsAg carriers with chronic hepatitis. This association could not be explained by confounding by age, the presence of HCV, or family history of HCC. The age-adjusted relative risk of 3.64 with 95% a confidence interval (1.34,9.90) is somewhat smaller than the estimated effect of aflatoxin exposure in male HBsAg carriers in Table 6 of Qian et al.,[3] namely 59.4/7.3 = 8.1. Both estimates are subject to substantial random error, however, and a formal test of heterogeneity, based on the crude counts in Table 6 of Qian et al.,[3] yields a χ^2 of 1.29 ($P = .26$). Thus, the data from these studies are consistent, even though the present study was conducted in a population of patients with chronic hepatitis, whereas Qian et al. studied the general male adult population in Shanghai.

Qian et al.[3] identified a subject as exposed to aflatoxin if any of the metabolites AFB1, AFP1, AFM1, or the DNA adduct, AFB1-N^7-Gua, were detected. They estimated an overall threshold for detection of aflatoxin of about 70 ng/L. Because we used samples of pooled urine that were 14 times larger than used by Qian et al., and because we also relied on a sensitive assay, we were able to detect as little as 3.6 ng/L of AFM1. To some extent, therefore, the lower relative risk that we observed compared with Qian et al. may reflect our inclusion of men with lower levels of AFM1 exposure, though as mentioned above, the difference may also be the result of chance alone. Our data were too sparse to demonstrate increasing risk of HCC with increasing levels of urinary AFM1 concentrations, however (Table 1).

Our data do demonstrate an association of AFM1 with persistent elevations of ALT in this population (Table 3), and there is a suggestion that AFM1 exposure increases the risk of fatal cirrhosis.

These data, which are based on a representative sample of men with chronic HBV hepatitis in Qidong, raise the possibility that programs to eliminate exposure to aflatoxin might benefit HBsAg carriers with chronic hepatitis. Indeed, the estimated attributable risk of 0.553 in this population suggests that about half the cases of HCC might be eliminated by reducing aflatoxin exposure to undetectable levels (less than 3.6 ng/L of AFM1 in urine). Ross et al.[2] had raised this possibility previously on the basis of studies in the general adult male population of Shanghai. To test this idea, one would first want to demonstrate that practical interventions can drastically reduce or eliminate aflatoxin exposure. This may prove difficult, because the aflatoxin intake in our study population was not high. Most of the 145 pooled urine samples had aflatoxin concentrations less than 100 ng/L, which is equivalent to the intake of less than 7,000 ng of aflatoxin daily.[4] Taking 65 kg as Qidong farmers' average body weight, the intake was below 110 ng/kg/d. This level is much lower than the median toxic dose for some rodents, the most sensitive species to aflatoxins, and humans are considered to be relatively resistant to aflatoxin, as are nonhuman primates.[10] Similar studies performed in the early 1980s, when corn was a major staple in Qidong, also demonstrated that urinary excretion of AFM1 was less than 100 ng/L in most cases.[5,11] If effective interventions were identified, however, one could consider the feasibility of randomized intervention trials in clinic-based populations. To detect a 2-fold reduction in HCC incidence rates with a power of 0.9 using a 2-sided log rank test would require following about 700 subjects for 5 years. Longer follow-up periods or larger cohorts would be needed to account for the fact that reducing aflatoxin exposure might not produce an immediate lowering in HCC risk.

Our finding of missense mutations in the third base of codon 249 in exon 7 of the p53 gene in the 4 specimens available from men with HCC and detectable AFM1 is consistent with and strengthens the findings in the literature that aflatoxin is characteristically associated with mutations at this codon.[6,7,12] Even though our data are limited, it appears that infection with HCV greatly increases the risk of HCC in men with chronic HBV hepatitis (Table 1), and this elevation in risk is not explained by confounding by age or by AFM1 exposure (Table 2). Ikeda et al.[13] found that patients with HCV hepatitis had a 15-year cumulative HCC incidence of 27%, compared with 19% for patients with HBV hepatitis. There were too few HCC events in patients with both HCV and HBV to gauge the joint effects of these viruses, however.

TABLE 3. Association of Persistently Elevated ALT with Urinary AFM1 >3.6 ng/L, Adjusted for HCV

	HCV + AFM1		HCV − AFM1		Total AFM1	
	>3.6 ng/L	≤3.6 ng/L	>3.6 ng/L	≤3.6 ng/L	>3.6 ng/L	≤3.6 ng/L
ALT						
Persistent elevation	3 (50%)	1 (25%)	40 (55%)	21 (33%)	43 (55%)	22 (33%)
No persistent elevation	3	3	32	42	35	45
Odds ratio	3.00		2.50		2.51 (crude)	
Adjusted odds ratio*					2.53	
Mantel-Haenszel χ^2					7.21 ($P = .007$)	

*Mantel-Haenszel method.

Case-control data from Taiwan[14] revealed 6 cases of HCC with both HBsAg and HCV-RNA positivity, compared with none among matched controls, suggesting that HCV may add to the strong effects of HBV exposure in this population. The Taiwan study was subject to selection biases, however, because cases and controls were selected from among members of a screened population who were positive for at least 1 of 6 risk factors, including the presence of HBsAg or antibody to HCV. Thus, even though our data are sparse, they may provide some of the strongest evidence that HCV increases the risk of HCC in men with HBV hepatitis. London et al.[15] studied a cohort of healthy men in Haimen City, China. They reported a relative risk of HCC of 2.4 (with a 95% confidence interval of 1.4-5.3) in men with a family history of HCC among first-degree relatives and a relative risk of 5.1 (1.5-14.4) in men with a family history of HCC among second-degree relatives. Though no data were presented, they reported that HBsAg status "did not affect the results" for family history and other factors studied. Our data, though sparse, indicate that men with chronic HBV hepatitis are at higher risk of HCC if they have a family history of HCC. In these data, 21 families had affected first-degree relatives, and 2 families had 2 affected uncles each. It is possible that this familial association is partly the result of early familial transmission of HBV, rather than of genetic factors. For example, if men with a family history of HCC are infected earlier with HBV, via familial transmission, than other men with HBV hepatitis, and if the hazard of HCC increases with the duration of HBV infection, then the association of family history with HCC risk may partly represent uncontrolled confounding from longer duration of HBV infection. To control for such a potential confounder, one would need information on the age at HBV infection.

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